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Polo-like kinase 1 expression is a prognostic factor in human colon cancer

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Abstract

AIM: To clarify the expression patterns and prognostic implications of the mitotic regulator Polo-like kinase 1 (PLK1) in colon cancer.

METHODS: Expression of PLK1 was investigated by immunohistochemistry (158 cases) and immunoblotting in tissue of colon adenomas and adenocarcinomas. PLK1 expression patterns were correlated with clinicopathological parameters and patient prognosis. In addition, expression of PLK1 was evaluated by immunoblot and PCR in colon carcinoma cell lines, and coexpression of PLK1 with the proliferation marker Ki-67 was investigated.

RESULTS: Weak PLK1 expression was observed in normal colon mucosa and adenomas. In contrast, 66.7% of carcinomas showed strong expression of PLK1. Overexpression of PLK1 correlated positively with Dukes stage ($P<0.001$), tumor stage ($P = 0.001$) and nodal status ($P<0.05$). Additionally, PLK1 expression was a prognostic marker in univariate survival analysis ($P<0.01$) and had independent prognostic significance (RR = 3.3, $P = 0.02$) in patients with locoregional disease. Expression of PLK1 mRNA and protein was detected in all cell lines investigated. Coexpression of PLK1 and Ki-67 was observed in the majority of colon cancer cells, but a considerable proportion of cells showed PLK1 positivity without Ki-67 expression.

CONCLUSION: PLK1 is a new prognostic marker for colon carcinoma patients and may be involved in tumorigenesis and progression of colon cancer. Strategies focusing on PLK1 inhibition *in vivo* might therefore represent a promising new therapeutic approach for this tumor entity.

Key words: Polo-like kinase; Colon carcinoma; Survival; Immunohistochemistry; Mitosis

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INTRODUCTION

Colorectal cancer ranks third in cancer deaths for both sexes in the Western world, with 146 940 estimated new cases and 56 730 estimated deaths in USA in 2004[1]. Incidence and mortality of colon cancer have decreased only slightly over the last 20 years. Though surgery is potentially curative, the risk of recurrence is high. Apart from surgical resection, treatment strategies for high risk patients are still mainly based on adjuvant/neoadjuvant use of 5-fluorouracil and leukovorin as chemotherapeutic agents alone or in combination with radiotherapy. Unfortunately, use of the standard therapeutic protocols results only in a moderate decline in mortality and the risk to sustain a recurrence of disease remains high[2]. Furthermore, it is still uncertain which of the patients should be treated, as prediction of the prognosis for an individual patient remains problematic[3]. With increasing knowledge about molecular changes in the malignant phenotype, therapeutic strategies focusing on targeted modulation of signal transduction pathways, which are imperative to malignant cells, are becoming increasingly important. Genetic alterations, typically associated with a malignant cell phenotype, affect genes involved in DNA repair and apoptosis, cell adhesion and invasion, angiogenesis and finally cell proliferation and cell cycle control[4].

After its discovery in Drosophila 16 years ago by Sunkel and Glover[5] *polo* has become the founding member of a whole family of protein kinases centrally involved in the mitotic regulation of both normal and malignant transformed cells. Since then, polo homologs have been discovered in a broad variety of species, including certain bacteria, yeast, mice, and men[6,7]. Till date, there are four known polo homologs in human beings with Polo-like kinase 1 (PLK1) being the best characterized protein of this family[8].

There is convincing evidence that PLK1 plays a central role in the G_2/M transition by exerting an important control function in several steps of mitosis[7]. Additionally, PLK1 plays an important role in the regulation of microtubule dynamics and in the maturation of centrosomes[9].

Expression of PLK1 has been described in a variety of

human malignancies[10-21]. We and others have reported that PLK1 overexpression had a significant impact on patient prognosis in some of these tumor entities[0,13,15,17,20]. For colon cancer, the prognostic impact of PLK1 has not been investigated so far.

The central aim of this study was to evaluate the status of PLK1 expression in a cohort of 158 benign and malignant colon tumors and in colon cancer cell lines by immunohistochemistry and immunoblotting, and to investigate the association of PLK1 expression with clinicopathological parameters and patient survival.

MATERIALS AND METHODS

Patients

A total of 153 patients (age: 31-86 years, median 65.45 years) who were diagnosed for colon cancer at the Institute of Pathology, Charité University Hospital between 1996 and 1999, were included in this study. Only patients with primary colon adenocarcinomas and no other known malignancies were included. None of the patients received radiotherapy or chemotherapy prior to diagnosis. All patients were residents of the city of Berlin. The majority of patients represented consecutive cases of colon cancer in our institute. Based on tissue availability in our archive, a small number of cases (7.8%, 12 cases) had to be excluded from this study. Histologic diagnosis was established on standard H&E stained sections according to the guidelines of the World Health Organization. The details on the distribution of clinicopathological factors in the study cohort are listed in Table 1. Clinical follow-up data were available for all patients. The median follow-up time of survivors was 47 mo. Forty-one patients (27%) died after a median time of 60 mo of follow-up. As a control for non-malignant colon tumors, five adenomas of the colon were included in the study as well.

Cell lines

The human colon carcinoma cell lines RKO and HT29 were obtained from ATCC. CX2 and HRT18 were from the German Cancer Research Center (Heidelberg, Germany). Cells were cultured in either DMEM (RKO) or RPMI (CX2, HRT18, HT29) supplemented with 10% fetal bovine serum.

Polymerase chain reaction

Subconfluent colon carcinoma cells were harvested and total RNA was prepared with RNAeasy kit (Qiagen, Hilden, Germany) and reverse transcribed. PCR cycling conditions for PLK1 were 30 cycles of denaturation, annealing and extension at 95 ℃ for 60 s, at 54 ℃ for 60 s, and at 72 ℃ for 120 s. The primers used were human PLK1 sense 5'-AGTGCTGCAGTGACTGCA-3' and antisense 5'-GGAGGCCTTGAGACGGTT-3' (generating a 1 807-bp band). As control GAPDH sense 5'-ACCACAGTCC-ATGCCATCAC-3' and antisense 5'-TCCACCACCCTG-TTGCTGTA-3' (generating a 452-bp band) primers were used.

Immunoblotting

Tissues from colon carcinomas and normal colon mucosa were dissected by a senior pathologist in the operation theater from surgical specimens sent for frozen section analysis and were immediately frozen in liquid nitrogen and stored at -80 ℃ until analysis.

Subconfluent colon carcinoma cells were harvested and 100 μg protein/sample was loaded on a 10% polyacrylamide gel. Western blots were performed as previously described[22], using a mouse monoclonal anti-PLK1 antibody (BD Transduction, San Diego, CA, USA) diluted 1:500 and an anti-β-actin antibody (Chemicon, Temecula, CA, USA) diluted 1:3 000.

Immunohistochemistry on tissue slides

For immunohistochemical detection of PLK1 on tissue samples and cells, a monoclonal mouse antibody (BD Transduction) directed against human PLK1 protein was used on 5-μm paraffin sections. Antibody specificity has been ascertained by Western blot and immunohistochemistry in preceding expression studies[14,23]. For immunolabeling of Ki-67, a polyclonal rabbit antibody (Dako, Glostrup, Denmark) directed against human Ki-67 protein was used. As negative controls, slides were processed without primary antibody. For antigen retrieval, the deparaffinized slides were placed in 0.01 mol/L sodium citrate buffer, pH 6.0 and boiled for 5 min in a pressure cooker. Then slides were allowed to cool down for additional 5 min in the same buffer. After several rinses in TBS and pre-treatment with blocking reagent (Dako, Glostrup, Denmark) for 5 min, slides were incubated with primary antibody diluted 1:500 (PLK1) and 1:50 (Ki-67) in antibody diluent solution (Zymed, San Francisco, CA, USA) for 20 min at room temperature and then at 4 ℃ overnight. After the slides were washed in TBS, bound antibody was detected by applying a streptavidin-biotin system (BioGenex, San Ramon, CA, USA) following the standard protocol with standard antibody dilutions as supplied by the manufacturers. For color development, a fast red system (Sigma, Deisenhofen, Germany) was used. The slides were mounted using Aquatex (Merck, Gernsheim, Germany).

Double-immunostaining of cells

RKO cells were grown on Labtek chamber slides. Subconfluent cells were fixed in 100% methanol for 20 min at -20 ℃. For immunolabeling of PLK1, slides were processed as described above but without applying the pressure cooker. After the color developed, slides were washed in PBS and subsequently incubated with a 1:50 dilution of primary antibody directed against Ki-67 for 20 min at room temperature and subsequently at 4 ℃ overnight. After the slides were washed in TBS, bound antibody was detected by the peroxidase anti-peroxidase method following the standard protocol with standard antibody dilutions as supplied by the manufacturers (Dako). Color development was performed using a DAB+system (Dako). Finally, slides were mounted using Aquatex.

Evaluation of tissue staining

Staining intensity of tissue slides was evaluated independently by two pathologists who were blinded towards patients' characteristics and survival. Cases with disagreement were discussed using a multi-headed microscope until agreement

was achieved. To assess differences in staining intensity, an immunoreactivity scoring system (IRS) was applied. Intensity of staining was designated as negative (0), weak (1), moderate (2) or strong (3). Additionally, the percentage of positive cells was evaluated and scored as either no cells (0), less then 10% of cells (1), 10-50% of cells (2), 51-80% of cells (3) or over 80% of cells stained (4). By multiplication of these two parameters, the IRS for each case was calculated. Finally, cases were grouped as PLK negative (IRS 0-6) or PLK positive (IRS 7-12) for statistical analysis.

Statistical analysis

Statistical correlation between clinicopathological factors and expression of PLK1 was assessed by applying either Fisher's exact test, Spearman's rank order correlation or χ^2 test for trends. The probability of differences in overall survival as a function of time was determined by Kaplan-Meier analysis and log-rank test. Multivariate probing for significance was performed applying the Cox proportional hazard model. Generally $P<0.05$ was considered statistically significant. For all statistical procedures, SPSS v10.0 software was used.

RESULTS

PLK1 expression in colon tissue and cell lines

Normal colon mucosa from both the vicinity of benign and malignant tumors as well as from more distant sites showed a weak cytoplasmic staining of the epithelium at the basis of colon crypts (Figure 1). Staining was lost in the epithelium of apical parts of the crypts. A comparable staining in the epithelium was observed on serial sections for the proliferation marker Ki-67 (data not shown).

Occasionally, lymphocytes resident in normal colon mucosa showed moderate staining for PLK1 in the cytoplasm. Moderate PLK1 positivity was observed in autonomous neural plexus cells in the submucosa and muscularis propria of the colon. This staining served as an internal positive control.

None of the five adenomas of colon mucosa showed sufficient staining of PLK1 to be designated as PLK1 positive, although a weak patchy staining in the epithelium was observed in all cases (Figure 1).

Among the 153 colon cancers investigated, 102 (66.7%) were PLK1 positive in the neoplastic epithelium (Figure 1 and Table 1). Staining was inhomogeneous in a considerable number of cases with pronounced PLK1 positivity at the leading edge of tumor invasion. However, cases scored as PLK1 negative consistently showed a weak irregular staining for PLK1 in the cytoplasm as well (Figure 1). The tumor-associated inflammatory infiltrate occasionally expressed moderate amounts of PLK1, while all other stromal cells were PLK1 negative in all cases.

Overexpression of PLK1 in colon carcinoma compared to normal colon mucosa could be confirmed by immunoblotting in three of four matched samples of normal colon mucosa and colon carcinoma (Figure 2). In one case the tumor tissue showed a loss of expression in comparison to the normal colon mucosa.

In addition to the investigation of primary colon carcinoma tissue, we also tested four colon carcinoma cell lines for expression of PLK1 mRNA and protein. As shown in Figure 2, all cell lines expressed PLK1 mRNA measured by RT-PCR with a 1 807-bp band. On the protein level, PLK1 expression with an estimated size of 68 ku was

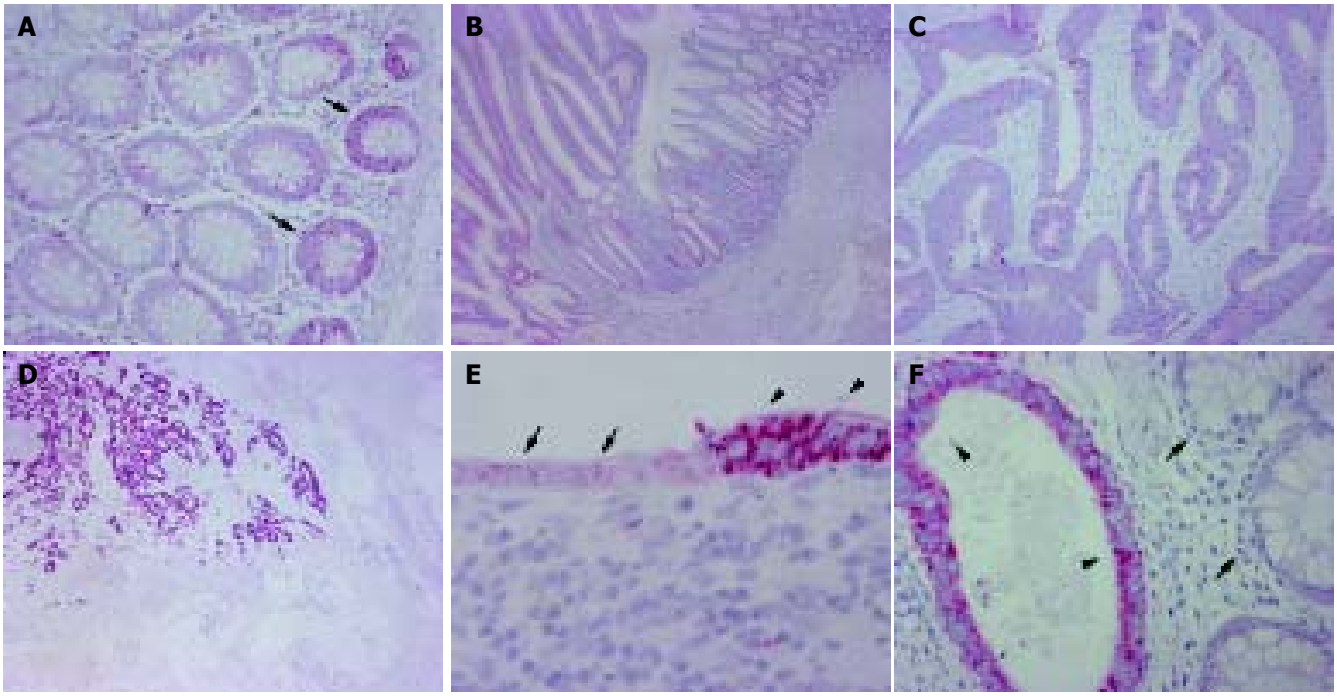

Figure 1 Expression of PLK1 in colon tissue. **A**: PLK1 expression in epithelium of the basis of normal colon crypts (arrows); **B**: Weak PLK1 positivity in colon adenoma; **C**: Weak expression of PLK1 in colon carcinoma; **D**: Strong positivity for PLK1 in colon carcinoma infiltrating pericolonic soft tissue; **E**: Transition of slightly dysplastic colon epithelium (small arrows) into colon carcinoma (arrowheads). Note the abrupt onset of PLK1 expression; **F**: Overexpression of PLK1 in colon carcinoma (arrowheads) infiltrating normal colon mucosa (small arrows). Original magnifications **A**: ×200, **B**: ×50, **C**: ×100, **D**: ×20, **E**/**F**: ×400.

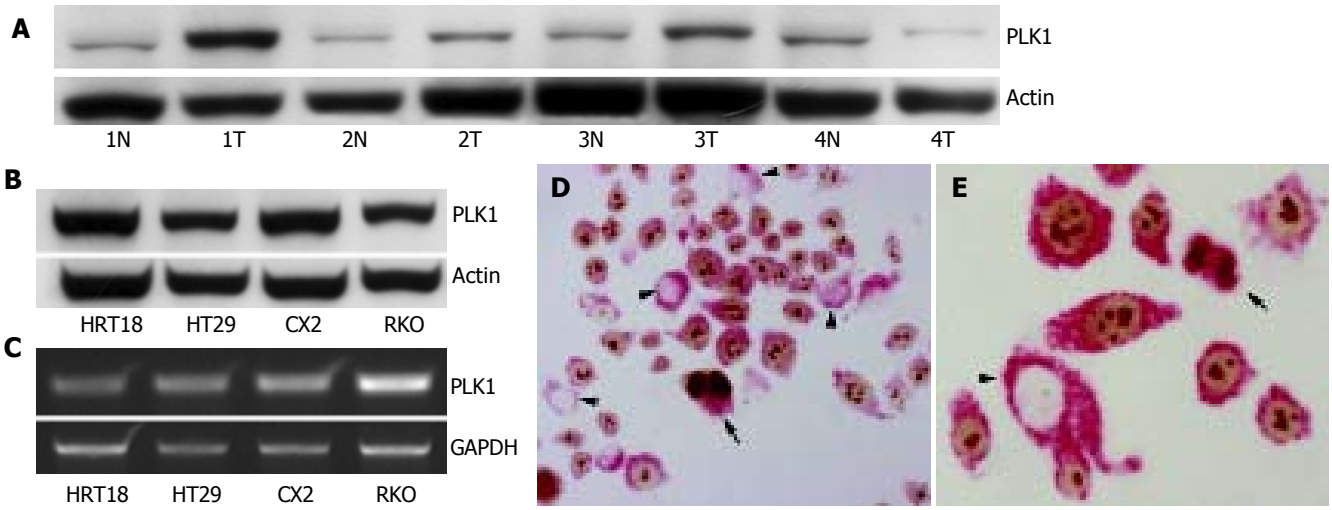

Figure 2 Expression of PLK1 in frozen colon tissue and colon carcinoma cell lines. **A**: Expression of PLK1 protein (68 ku) in colon carcinoma and normal colon mucosa; **B**/**C**: Strong expression of PLK1 protein (**B**, immunoblot, 68 ku) and mRNA (**C**: RT-PCR, 1 807 bp) in colon carcinoma cell lines; **D**/**E**: Coexpression of PLK1 (red staining of the cytoplasm) and Ki-67 (brown staining of the nucleus) in RKO colon carcinoma cell line. A considerable number of cells were PLK1 positive, but remained Ki-67 negative (arrowheads). Note cells in M-phase (small arrows) strongly expressing both proteins. Original magnifications **D**: ×200, **E**: ×500.

detected in all cell lines by immunoblot.

PLK1 and Ki-67 coexpression in primary colon carcinomas and RKO cell line

Since PLK1 plays a pivotal role in cell cycle control, we investigated the coexpression of Ki-67 and PLK1 in the colon cancer cell line RKO by immunolabeling both proteins consecutively on the same slide. As illustrated in Figure 2, simultaneous expression of PLK1 in cytoplasm and Ki-67 in nuclei was evident in the majority of cells. However, while almost all cells expressed PLK1, there was a considerable fraction of cells without simultaneous Ki-67 expression. Similar results were observed in serial sections of primary colon carcinoma tissue. Staining of serial sections of colon carcinomas with PLK1 and Ki-67 revealed no strict overlap between the expressions of both proteins though highly proliferating tumors (strong Ki-67 positivity) were more prone to overexpress PLK1.

Correlation of PLK1 expression with clinicopathological data

A highly significant positive correlation of PLK1 expression with either Dukes stage ($P<0.001$, χ^2 test for trends) and WHO tumor stage ($P = 0.001$, χ^2 test for trends) could be observed. More advanced tumors expressed significantly higher amounts of PLK1 than locally restricted carcinomas (Table 1). Additionally, a significant positive correlation of PLK1 expression with nodal status could be demonstrated ($P = 0.049$, χ^2 test for trends). This observation was further confirmed by a significant positive correlation ($P = 0.044$, $r = 0.163$, Spearman's rank order correlation) of the number of positive lymph nodes with ungrouped PLK1 score. We did not observe a correlation of PLK1 expression in colon cancer with patients' age, status of distant metastasis and tumor grade (Table 1)

Correlation of PLK1 expression with patient prognosis

Known prognostic parameters in colon cancer, as patients' age, Dukes stage, WHO tumor stage, lymph node status, status of distant metastasis and tumor grade were confirmed to have a significant impact on patient prognosis in this study (Table 2).

Additionally, PLK1 expression had a significant impact on patient prognosis both in the whole study cohort ($n = 153$, $P = 0.006$) and in the subgroup of patients without distant metastasis at the time of diagnosis ($n = 140$, $P = 0.012$). The mean survival time of patients in the PLK1 negative groups was 74.13 mo (whole group) and 76.36 mo (subgroup without distant metastasis), while the survival time was reduced to a mean of 63.75 mo (whole group) and 67.77 mo

Table 1 Overall expression of PLK1 in colon carcinoma as well as distribution of PLK1 expression in the study population, *n* (%)

Characteristics		All cases	PLK1 negative	PLK1 positive	*P*
All cases		153	51 (33.3)	102 (66.7)	
Age (yr)					
	≤65 yr	74 (48.4)	28 (37.8)	46 (62.2)	0.304[1]
	>65 yr	79 (51.6)	23 (29.1)	56 (70.9)	
Dukes stage					
	A	35 (22.9)	20 (57.1)	15 (42.9)	
	B	55 (35.9)	19 (34.5)	36 (65.5)	<0.001[2]
	C	50 (32.7)	10 (20)	40 (80)	
	D	13 (8.5)	2 (15.4)	11 (84.6)	
Tumor stage					
	T1	10 (6.5)	9 (90)	1 (10)	
	T2	36 (23.5)	14 (38.9)	22 (61.1)	0.001[2]
	T3	96 (62.7)	24 (25)	72 (75)	
	T4	11 (7.3)	4 (36.4)	7 (63.6)	
Nodal status					
	N0	91 (59.5)	38 (41.8)	53 (58.2)	
	N1	30 (19.6)	4 (13.3)	26 (86.7)	0.049[2]
	N2	32 (20.9)	9 (28.1)	23 (71.9)	
State of distant metastasis					
	Mx	140 (91.5)	49 (35)	91 (65)	0.221[1]
	M1	13 (8.5)	2 (15.4)	11 (84.6)	
Grade					
	G1	6 (3.9)	2 (33.3)	4 (66.7)	
	G2	123 (80.4)	43 (35)	80 (65)	0.423[2]
	G3	24 (15.7)	6 (25)	18 (75)	

[1]Fisher's exact test, [2]χ^2 test for trends.

(subgroup without distant metastasis) in the PLK1 positive groups, respectively (Figure 3). In the whole study cohort, patients with PLK1 positive tumors showed a rate of 65% survival after 5 years compared to 86% survival in the PLK1 negative group. In the subgroup without distant metastasis, the 5-year survival was reduced from 89% in the PLK1 negative group to 70% in the PLK1 positive group (Table 2).

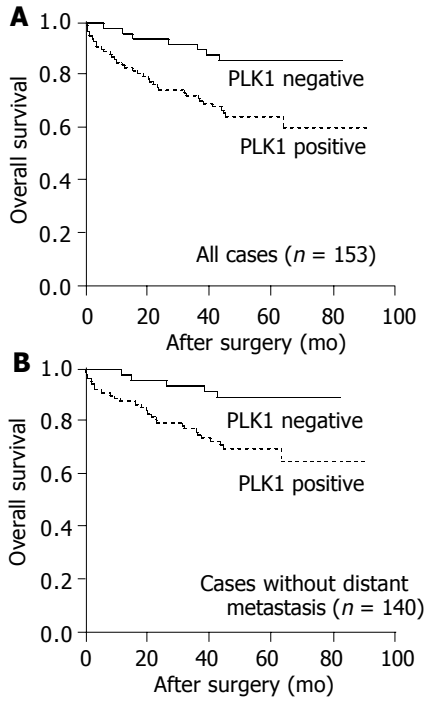

Figure 3 Correlation of PLK1 expression with patient survival in the whole study population (**A**) and in the subgroup of patients with locoregional disease (**B**).

Table 2 Correlation of PLK1 expression and selected tumor parameters with patient prognosis

		Cases	Events	Mean survival (mo)	Standard error	Log-rank test *P*
Age (yr)	≤65 yr	74	13	76.59	3.42	0.0142
	>65 yr	79	29	57.94	3.51	
Dukes stage	A	35	4	70.27	3.18	<0.0001
	B	55	5	83.89	2.67	
	C	50	23	48.26	4.28	
	D	13	10	22.96	6.60	
Tumor stage	T1	10	0	Not reached	–	0.0006
	T2	36	8	74.08	5.06	
	T3	96	27	63.84	3.06	
	T4	11	7	27.00	7.22	
Nodal status	N0	91	11	81.93	2.34	<0.0001
	N1	30	10	55.94	5.17	
	N2	32	21	28.74	3.97	
Metastasis	Mx	140	32	73.19	2.68	<0.0001
	M1	13	10	22.96	6.60	
Grade	G1	6	0	Not reached	–	0.0007
	G2	123	29	72.61	2.90	
	G3	24	13	33.37	4.68	
PLK1 (all cases)	Negative	51	7	74.13	2.90	0.0060
	Positive	102	35	63.75	3.66	
PLK1 (locoregional disease)	Negative	49	5	76.36	2.52	
	Positive	91	27	67.77	3.66	0.0119

In the whole study population, PLK1 expression failed to be a significant independent predictor of prognosis ($P = 0.091$, RR = 2.1). Independent prognostic predictors were patients' age, nodal status, state of distant metastasis and tumor grade (Table 3). In contrast, in the subgroup of patients without distant metastasis, PLK1 expression had a significant impact on patient prognosis in multivariate survival analysis. Patients with PLK1 positive tumors were approximately thrice (RR = 3.3, $P = 0.019$) more likely to die in the given time interval compared to their PLK1 negative counterparts. Again, patients' age, nodal status and tumor grade remained independent significant predictors of prognosis (Table 4).

Table 3 Multivariate survival analysis for all cases ($n = 153$)

	RR	95%CI	*P*
Age (yr)			
per yr	1.040	1.008–1.072	0.013
Tumor stage			
T1/T2	1.000		
T3/T4	1.061	0.466–2.413	0.888
Nodal status			
Per positive node	1.116	1.037–1.201	0.003
Metastasis			
Mx	1.000		
M1	3.223	1.202–8.643	0.020
Grade			
G1/G2	1.000		
G3	2.773	1.382–5.564	0.004
PLK1 expression			
Negative	1.000		
Positive	2.067	0.890–4.800	0.091

Table 4 Multivariate survival analysis for the subgroup of patients without distant metastasis ($n = 140$)

	RR	95%CI	*P*
Age (yr)			
per yr	1.044	1.009–1.079	0.013
Tumor stage			
T1/T2	1.000		
T3/T4	0.898	0.365–2.206	0.814
Nodal status			
Per positive node	1.231	1.125–1.348	<0.001
Grade			
G1/G2	1.000		
G3	2.614	1.172–5.829	0.019
PLK1 expression			
Negative	1.000		
Positive	3.306	1.214–9.002	0.019

DISCUSSION

To our knowledge, this is the first study showing an adverse effect of PLK1 overexpression on the prognosis of patients with colon cancer. This effect remains independently significant in the clinically important subgroup of patients without known distant metastasis.

In the present study, PLK1 was overexpressed in 66.7% of colon cancers and overexpression was positively linked to tumor stage and lymph node status, two parameters

that determine the extent of tumor burden. This is in line with the results of Takahashi *et al*.[21]. In another study by Macmillan *et al*.[24], increased PLK1 transcription levels were observed in colon carcinoma.

PLK1 overexpression in malignancies seems to be a common event, since it has been observed in a variety of different neoplasms, including cancer of stomach[10], esophagus[10], breast[11], endometrium[12], ovary[13], brain[14], head and neck[15], skin[16], lung[17], prostate[18], thyroid[19], liver[20], and colon[21]. Effects of PLK expression on patient prognosis have already been reported in a variety of other tumor entities, including esophageal carcinoma[10], ovarian carcinoma[13], carcinoma of head and neck[15], non-small cell lung carcinomas[17], and hepatoblastoma[20].

Among the possible functions of PLK1 overexpression in tumor biology, the most important factor might be the central role of PLK1 in mitotic regulation. PLK1 initiates mitosis by the activation of cdc25 and cyclin B1 via direct phosphorylation. Both events lead to an accumulation of cdc2/cyclin B1 complex in the nuclei, a precondition known to be crucial for the onset of mitosis[25,26]. Later in mitosis, PLK1 triggers the dissociation of cohesins from their chromosomal binding sites[27] and activates the anaphase-promoting complex[28]. The central involvement of PLK1 in mitosis of malignant cells is supported bys findings in several tumor entities including colon cancer[21], showing that PLK1 expression correlated positively with markers of cell proliferation.

Nevertheless, there are several arguments supporting the theory that additional functions of PLK1 must exist. First, only a part of the variety of PLK1 expression can be explained by mitotic activity. Second, we observed expression of PLK1 in irreversibly postmitotic cells such as neuronal ganglion cells. Third, PLK1 expression is unlikely to reflect cell proliferation exclusively, since we observed cases with strong PLK1 expression in almost 100% of tumor cells (Figure 1), a number that by far exceeds the rate of cells that can be expected to be in G_2/M phase. Finally, we could show that PLK1 expression is not strictly confined to cycling cells by double staining of colon cancer cells. Additional functions of PLK1 in tumor biology may have something to do with its ability to control microtubule dynamics[9], which is not only essential in mitosis but also important in golgi protein transport and cytoskeletal formation and rearrangement. These possible additional roles of PLK1 overexpression in tumor biology should be subjected to further functional studies.

Another explanation for the cause of PLK1 deregulation might be that either enhanced transcription by altered intracellular signal transduction or chromosomal over-representation of the respective gene locus leads to high cellular protein levels, which in turn enhance mitotic activity. In fact, chromosomal amplifications of 16p12, the gene locus of PLK1 has been described in human colorectal carcinomas[29].

Most recently, several groups found that inhibition of PLK1 expression in cancer cells of various tumor entities leads to a dramatic decline in cell proliferation[23,30] and to an induction of apoptosis[31]. These results suggest that PLK1 is an attractive target for novel chemotherapeutic approaches. Expression studies, like ours, may thus provide the translational basis for such new therapeutical approaches.

In summary, PLK1 is overexpressed in 66.7% of colon cancers, overexpression of PLK1 is linked to advanced tumor stages in this disease and can be considered as a new independent prognostic marker for colon cancer patients with locoregional disease.

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